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LETTER TO THE EDITOR

The Issue of Skeletal Muscle Growth and Regeneration

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Comment on:
PETER MAKOVICKÝ, PAVOL MAKOVICKÝ: Histological aspects of skeletal muscle fibers splitting of C57BL/6NCrl mice. *Physiol Res* **69**: 291-296, 2020

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Dear Editor,

Skeletal muscle reparation and regeneration is indeed a very interesting issue from various perspectives. The interest of both the expert communities and lay public is explicable by the issue of skeletal muscle injuries in professional athletes, one of the leading causes of prolonged discontinuation of their active sport career. Comparably interesting is also the area of research focused on genetically determined muscular atrophies. For the above-mentioned reasons, we have read with great interest the paper "Histological aspects of skeletal muscle fibers splitting of C57BL/6NCrl mice" by Makovický and Makovický (2020).

What grabbed our attention the most is that the manuscript title refers to "muscle fibers splitting". However, the photomicrographs in the presented article show clearly distinguishable muscle spindles, which are normal components of the skeletal muscle. However, this is not the only problem of this paper.

Mammalian skeletal muscle is capable of regeneration, although this ability has its limits if the skeletal muscle injury is extensive and a certain regenerative threshold is reached (Liu *et al.* 2018). Muscle regeneration is based predominantly on the action of satellite cells (also called resident muscle stem cells) and is mainly controlled through the expression of extracellular matrix (ECM) proteins and various bioactive molecules. Satellite cells are a heterogeneous population of quiescent cells, which are arrested at an early stage of the myogenic program (Oprescu *et al.* 2020). In case of injury, they are activated by the effect of specific molecules (e.g. MRF4, myogenin, MyoD, and Myf5), they proliferate and undergo the process of differentiation into myoblasts (Zammit 2017). Consequently, myoblasts fuse with each other to produce multinucleate myotubes, which give rise to mature muscle fibers. The plasticity of ECM is imperative to the ability of satellite cells to become activated, for their differentiation and subsequent migration to the location of injury (Petrosino *et al.* 2019). The paper by Murach *et al.* (2019), also cited by the authors, suggests that muscle fiber splitting may occur physiologically as a satellite cell-independent process, however, largely as a response to extreme overload of a muscle. We have not found any mention of such approach applied to mice described by Makovický and Makovický (2020).

The principal problem and the main flaw of the

article by Makovický and Makovický (2020) is that in the slides from murine limb skeletal muscle the authors failed to identify muscle spindles and confused them with dividing skeletal muscle fibers. Muscle spindles are present in large numbers in skeletal muscles and are the most frequently found sensory organs in the musculoskeletal tissues of mammalian limbs (Ellaway *et al.* 2015). Each individual skeletal muscle of the limb contains 25-114 muscle spindles (Banks 2006). Just as a matter of interest, we would like to add that human skeletal muscles contain 44 000 muscle spindles in total (Voss 1971). It is not surprising, from the perspective of various functional roles of skeletal muscles, that each muscle should possess a characteristic proprioceptive innervation. Muscle spindle *(fusus neuromuscularis)* is the spindle-shaped intramuscular stretch receptor which is important in the regulation of muscle contraction. A single muscle spindle receives one or more sensory nerve fibers, whose endings are located more or less in the middle of a small bundle of specialized intrafusal muscle fibers. These intrafusal fibers also receive their own motor innervation, allowing for the phasic and tonic aspects of the sensory responses to be independently adjusted (Bewick and Banks 2015).

The microscopic structure of the muscle spindles in clearly visible in the photomicrographs published in the paper by Makovický and Makovický (2020), especially their Fig. 1 C-F contain typical examples of muscle spindles. For a comparison, we provide our own photomicrographs (our Fig. 1A-B). The muscle spindles are located inside the skeletal muscle and are surrounded by thin connective tissue capsule comprised of fibroblasts and delicate collagen fibers. After more precise examination, inner and outer layer (internal and external lamina) can be distinguished with a space between them filled with glycosaminoglycan-containing jelly-like fluid. The muscle spindle contains intrafusal muscle fibers *(myofibrae intrafusales)*, which differ from the regular muscle fibers in several features: they are shorter and they have fewer myofibrils. Nuclear bag fibers have aggregated nuclei occupying the central region. Nuclear chain fibers possess multiple nuclei arranged in chains. The polar region contains muscle fibers with motor end plates, while the equatorial region contains annulospiral sensory nerve endings. Apparently, this typical microscopic structure, which is illustrated in most pregraduate histology textbooks (Balko *et al*. 2018, Ross and Pawlina 2016) as well as in histopathology manuals (Heffner and Balos 2007), was confused with the process of muscle fiber splitting in the paper by Makovický and Makovický (2020). Many scientific papers dealing with mammalian muscle spindles in more detail are available, demonstrating e.g. the spatial reconstruction, fiber typing, histochemistry, and electron microscopy of the intrafusal fibers (Thornell *et al.* 2015), their innervation patterns (Banks 2015), fusimotor activity (Ellaway *et al*. 2015), and more.

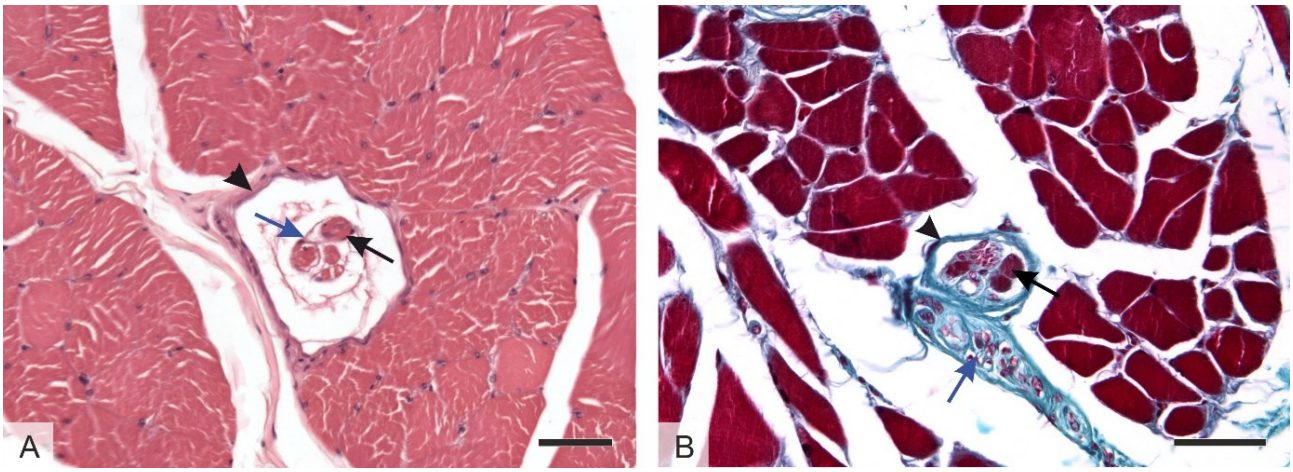

Fig. 1. Examples of mammalian muscle spindles as shown in routine preparations of skeletal muscles used in pregraduate Histology courses. (**A**) These stretch receptors are surrounded by an external capsule (black arrowhead). The internal capsule (blue arrow) contains intrafusal muscle fibers (black arrow). Interosseal muscles of the cat metacarpus, hematoxylin eosin stain, scale bar 50 μm. (**B**) External capsule (black arrowhead), intrafusal muscle fibers (black arrow), and unmyelinated nerve fibers. Human tongue, Verhoeff's iron hematoxylin and green trichrome stain, scale bar 50 μm.

In the following points, we add several other comments, which put the finishing touches to the complex picture of this scientific article:

- "*Methods*: The morphometry results were analyzed using …" The results don´t contain any morphometry. What exactly did the authors measure? How was the countable event defined? How was the edge effect eliminated? What sampling strategy was applied? Did the authors quantify the diameter of muscle fibers, size of the nuclei, ratio between muscle tissue and interstitial connective tissue? Neither quantitative methods nor tools were described, what made the effort non-reproducible. As the authors probably counted various phenomena, the study cannot even be used for mapping the muscle spindles within the mouse rectus femoris muscle (Sato *et al.* 2007). Tschanz *et al.* (2014) summarized all the necessary requirements for planning, designing, and performing a successful morphometric study.
- "*Results*: …hypertrophic spherical shape basophilic skeletal muscle fibers…" Skeletal muscle fibers are always acidophilic, never basophilic. This is due to the high content of mitochondria, myoglobin and smooth endoplasmic reticulum.
- "*Results*: There is skeletal muscle fiber hypertrophy with nuclei movement at their periphery". Every skeletal muscle fiber has its nuclei located at the periphery. Moreover, without any scale bar, it is very hard for a reader to notice which muscle fiber is hypertrophic. No definition of hypertrophy was provided in the paper. Moreover, no reasons were provided why the fibers should undergo hypertrophy since all the mice were kept in similar environment with similar conditions, probably without any extreme physical activity.
- "*Results*: Part of the split skeletal muscle fiber is phagocytosed …" This claim by the authors is not documented anywhere in the paper, even though the demonstration of macrophage presence is routinely performed, e.g. using antibodies against MAC 387 or CD68 or other monocyte/macrophage immune-histochemical markers. No macrophages are shown in the routine sections either.
- "*Results*: Splitting skeletal muscle fibers..." No markers of cell division and proliferation were used at all, although the study refers to "skeletal muscle regeneration".
- *Results*: The nuclei move from the periphery to the center of a split skeletal muscle fiber". Centrally located nuclei are found only in intrafusal muscle fibers inside the muscle spindles. Due to the main methodology flaw, the authors' description could probably match some of the intrafusal bag fibers with central aggregation of nuclei.
- "*Results*: Such skeletal muscle fibers are well recognizable due to the presence of bright, vesicular nuclei with prominent nucleoli … (Fig. 1D)". The photomicrographs do not correlate with the narrative description of the results. With the magnification so low, and the pictures so blurred, it is hard to distinguish cell nuclei and almost impossible to distinguish mentioned nucleoli. Unfortunately, none of the presented photomicrographs display any of the "unique" findings the authors have observed, as they claim in the main text. In each picture, we only see muscle spindles, not dividing muscle fibers.
- "*Results*: … junctions between peripheral nerves and skeletal muscle fibers visible with focal axon degeneration". What criteria of axonal degeneration were applied? How have the authors managed to observe focal axonal degeneration under a light microscope by using only such low magnifications and on top of that, by examining slides stained only with hematoxylin and eosin?
- "*Results*: No significant differences in the average percentage of skeletal muscular fiber regeneration, average percentage of hypertrophic skeletal muscular fibers …" What method was used by the authors to study muscle fiber regeneration, which, under normal conditions, occurs via the activation of satellite cells? How have they managed to identify which muscle fibers were hypertrophic, when they have not mentioned any normative? These results are probably imaginative and not substantiated by own genuine research. No primary data are presented in the Results either in a graphical or in tabular form.

The aim of our criticism of the paper authored by Makovický and Makovický (2020) was to prevent the readers from being misinformed by a paper based on a major flaw such as confusing splitting muscle fibers with a stretch receptor.

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